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Antidepressant-like effects of BU10119, a novel buprenorphine analogue with mixed kappa/mu opioid receptor antagonist properties, in mice

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Abbreviations: CCAM – clocinnamox; CPP – conditioned place preference; κ -receptor - kappa opioid receptor; EPM – elevated plus maze; LDB – light-dark box; %MPE -percentage maximum possible effect; μ -receptor – mu opioid receptor; NOP receptor – nociception/orphanin FQ receptor; norBNI- norbinaltorphimine

Author Contribution:

A. Almatroudi: PhD student who performed all of the experiments and data analysis, first draft of the manuscript.

M. Ostovar: Postdoctoral researcher who synthesized BU10119 for these experiments.

C.P. Bailey: Contributed to the experimental design, research and drafting of the manuscript.

S.M.Husbands: Contributed to the experimental design, research and drafting of the manuscript.

S.J.Bailey: Contributed to the experimental design, research and lead author on drafting the manuscript.

Competing Interests' Statement: None

Abstract

Background and Purpose

K-opioid receptor antagonists have potential for treating neuropsychiatric disorders. We have investigated the *in vivo* pharmacology of a novel buprenorphine analogue, BU10119, for the first time.

Experimental Approach

To determine the opioid pharmacology of BU10119 (0.3-3 mg/kg, i.p.) *in vivo*, the warm-water tail withdrawal assay was used in adult male CD1 mice. A range of behavioural paradigms was used to investigate the locomotor effects, rewarding properties and antidepressant or anxiolytic potential of BU10119. Additional groups of mice were exposed to a single (1 x 2h) or repeated restraint stress (3 x daily 2h) to determine the ability of BU10119 to block stress-induced analgesia.

Key Results

BU10119 alone was without any antinociceptive activity. BU10119 (1mg/kg) was able to block U50,488, buprenorphine and morphine induced antinociception. The κ -antagonist effects of BU10119 in the tail-withdrawal assay reversed between 24-48h. BU10119 was without significant locomotor or rewarding effects. BU10119 (1mg/kg) significantly reduced the latency to feed in the novelty-induced hypophagia task and reduced immobility time in the forced swim test, compared to saline treated animals. There were no significant effects of BU10119 in either the elevated plus maze or the light dark box. Both acute and repeated restraint stress-induced analgesia was blocked by pretreatment with BU10119 (1mg/kg). Parallel stress-induced increases in plasma corticosterone were not affected.

Conclusions and Implications

BU10119 is a mixed κ/μ -opioid receptor antagonist with relatively short-duration κ -antagonist activity. Based on these preclinical data, BU10119 has therapeutic potential in the treatment of depression and other stress-induced conditions.

Introduction

There is growing interest in the possibility of targeting kappa opioid receptors (κ -receptors Borsodi et al. 2017) for the treatment of depression (Carroll and Carlezon, 2013, Lalanne et al., 2014). The endogenous opioid neuropeptide dynorphin is released in response to stress and activates κ -receptors to produce pro-depressive behaviours (Shirayama et al., 2004, Schwarzer, 2009). In humans, activation of κ -receptors has been shown to be dysphoric (Pfeiffer et al., 1986). On the other hand, blockade of κ -receptors or κ -receptor gene deletion have shown anti-depressant like effects in mice (McLaughlin et al., 2006b, McLaughlin et al., 2003).

A number of high affinity, selective κ -antagonists exist (Carroll and Carlezon, 2013) which have an unusual pharmacokinetic profile that perhaps limits their translatability to the clinic. For example, following a single injection, the effects of the selective κ -receptor antagonist norbinaltorphimine (norBNI) last for several weeks (Endoh et al., 1992). A number of strategies have arisen to develop alternative means of targeting the κ -receptor by producing short-acting κ -antagonists (Aldrich et al., 2009, Rorick-Kehn et al., 2014, Casal-Dominguez et al., 2014, Peters et al., 2011, Verhoest et al., 2011, Ross et al., 2012).

We have recently shown that the combination of buprenorphine (a partial μ opioid receptor agonist/ κ -antagonist) with naltrexone (a non-selective μ/κ antagonist) produces a functional short-acting κ -antagonist *in vivo* that has antidepressant-like activity in mice (Almatroudi et al., 2015). A similar approach of a combination of buprenorphine with a μ -antagonist samidorphan has been shown to have

antidepressant effects in treatment resistant depressed patients (Ehrich et al., 2015). While buprenorphine and naltrexone are both licensed for other indications, so may be attractive to translate to the clinic, the approach of using combination treatments may have limitations. Where buprenorphine/naltrexone combination has been trialled clinically for the treatment of opioid dependence (Gerra et al. 2006, Rothman et al. 2000), naltrexone was administered orally and buprenorphine sublingually, so achieving the correct dose combination to achieve an antidepressant effect may not be trivial (Almatroudi et al., 2015, Cordery et al., 2014). Furthermore, the risk of diversion of buprenorphine and its abuse liability as a partial μ -opioid receptor agonist must be considered. The non-therapeutic mis-use of buprenorphine has recently been reported to be rising among drug users, as it serves as a substitute for other drugs of abuse (Cicero et al., 2014).

An alternative strategy to targeting κ -receptors, building on this combination approach, is to design novel chemical entities that combine the pharmacology of buprenorphine and naltrexone into one molecule. This would overcome both the abuse liability issue and the dosing issues. BU10119 is one of a novel series of orvinol analogues in which the C20-methyl group has been moved to the C7-position (Cueva et al., 2015, Figure 1). In vitro pharmacology studies have established that BU10119 has high affinity for both κ and μ receptors with little efficacy at either of these receptors indicating an antagonist like profile (Cueva et al., 2015, Table 1). Here we report the initial characterization of BU10119 *in vivo* and behavioural studies that demonstrate the therapeutic antidepressant-like potential in mice.

506/500 words

Table 1

Maximal Stimulation of [35S]GTP γ S binding of buprenorphine and BU10119 to opioid and NOP receptors

Compound	κ	μ	NOP
Buprenorphine (1a*)	0 \pm 6% (Ke = 0.14 \pm 0.06 nM)	20 \pm 6% (EC50: 0.7 \pm 0.3nM)	39 \pm 12% (EC50: 1480 \pm 980nM)
BU10119 (15a*)	-2 \pm 1% (Ke = 0.09 \pm 0.04 nM)	2 \pm 4% (Ke = 0.28 \pm 0.04 nM)	56 \pm 1% (EC50: 147 \pm 33nM)

Percent maximal stimulation at a single high concentration (10 μ M) with respect to the standard agonists U69,593 (κ), DAMGO (μ) and nociception (NOP). Agonist EC50 (nM) or antagonist Ke (nM) the antagonist dissociation constant determined against the same agonists. Neither buprenorphine nor BU10119 has any appreciable efficacy at δ -receptors. All data taken from Cueva et al. 2015.

Methods

Drugs

BU10119 was synthesised and supplied at the University of Bath (Cueva et al., 2015). Buprenorphine hydrochloride and morphine sulphate were purchased from MacFarlan Smith (Edinburgh, UK). U50,488 was obtained from Sigma (Dorset, UK). Clocinnamox mesylate (CCAM) and norBNI dihydrochloride were supplied by Tocris Bioscience (Bristol, UK). Fluoxetine hydrochloride and naltrexone hydrochloride were purchased from Abcam Biochemicals (Cambridge, UK). All drugs were injected via the intraperitoneal route at a volume of 10 ml/kg, except CCAM administered at 20ml/kg, and were dissolved in 0.9% w/v saline (Hameln Pharmaceuticals, Gloucester, UK).

Animals

Adult male CD-1 mice weighing 25 to 43 g (8–10 weeks) were used throughout these experiments. CD-1 mice were initially from Charles River (CrI:CD1(ICR)) and bred in-house at the University of Bath for more than 10 years. At weaning, mice were housed as mixed litter groups of 4 to 5 in cages (30 x 16 x 14 cm) with wood shavings and nesting material and with ad libitum access to food and water. Mice were maintained on a 12 h light-dark cycle (lights on 07:00 hours, lights off 19:00 hours) at $20 \pm 2^{\circ}\text{C}$. All experiments were performed in accordance with the UK Home Office guidelines, including local ethical review, and the Animals (Scientific Procedures) Act 1986/ Directive 2010/63/EU. Mice were randomly assigned to treatment groups. All behavioural experiments were performed between 09:00 and 16:00 hours by a male experimenter (Sorge et al., 2014) and mice were habituated

to the behavioural room for 1 h before starting an experiment. For each behavioural task separate groups of animals were used (n=5-18 per treatment group).

Warm water tail-withdrawal test

The warm water tail-withdrawal assay was carried out as described previously (Almatroudi et al., 2015). Water temperature was maintained at 52°C and the latency for tail withdrawal recorded. A 15-second cut-off was imposed to avoid tissue damage and antinociception calculated as percentage maximum possible effect (%MPE) = (test latency–control latency)/(15 s–control latency) ×100. To counteract any possible confounding effects of injection induced stress, in all experiments, animals received 0.9% w/v saline injections so that the total number of injections an individual mouse received, whether in control or in drug treated groups, was equivalent.

To examine the duration of the κ -antagonist properties of BU10119 (0.3, 1 and 3 mg/kg), tail withdrawal latencies were determined at intervals 1-48 h post-injection, with antinociceptive agonist challenge (U50,488) administered 30 min prior to each time point (Figure 2B). The μ -antagonist properties of BU10119 (0.3, 1 and 3 mg/kg) were established by blockade of the μ -agonist induced antinociceptive effects of buprenorphine (1 mg/kg) and morphine (10 mg/kg), and compared to the irreversible, selective μ -antagonist CCAM (3 mg/kg) (Broadbear et al., 2000) (Figure 2C).

Baseline latencies were measured immediately before injecting BU10119 or saline at time zero. Buprenorphine or morphine were injected 30 min later and 1 h elapsed before “test” tail-withdrawal latencies were measured. CCAM was injected 24 h before time zero (Broadbear et al., 2000)

To assess stress-induced analgesia, baseline latencies were measured before drug injection on the first and third day of restraint. These baseline latencies did not change across the duration of the experiment (Supplementary figure 1). BU10119 (1 mg/kg) or buprenorphine/naltrexone (1 mg/kg) combination was given daily 1h before restraint (naltrexone (1 mg/kg) was injected 10 minutes prior to buprenorphine). Tail-withdrawal latency was measured 5 minutes after the end of the restraint session on the first and third day. For mice treated with norBNI (10 mg/kg), the drug was given once only 24 h before the first restraint session.

Locomotor activity

To investigate any potential sedative effect of BU10119 (0.3, 1 mg, and 3 mg/kg), locomotor activity was assessed 1 h post-administration in a 10 min open-field test (72 x 72 cm) under low light conditions (30 lux) via infrared photobeams (Almatroudi et al., 2015).

Forced swim test

Antidepressant-like effects of BU10119 (1 mg/kg), buprenorphine/naltrexone combination (1 mg/kg) and the selective serotonin reuptake inhibitor fluoxetine were investigated in a 6 min forced swim test (Almatroudi et al., 2015). All drugs were administered 1 h prior to testing and the behaviour during the last 4 minutes of the test reported.

Novelty-induced hypophagia

The novelty-induced hypophagia paradigm used was as previously described (Almatroudi et al., 2015). Mice were trained on 3 consecutive days to consume

condensed milk. The latency to drink milk was recorded on day 4 in the home cage and on day 5 in a novel cage environment. Mice received fluoxetine (20 mg/kg), BU10119 (1 mg/kg) and buprenorphine/naltrexone combination (1 mg/kg) (naltrexone was injected 10 minutes prior to buprenorphine) 1 h prior to testing. For mice receiving the κ -receptor antagonist norBNI (10mg/kg), the drug was injected immediately after training on day 3, 24-48 h prior to testing.

Elevated plus maze (EPM)

The time spent in, and entries into, the open and closed arms, and total ambulation during a 5 min EPM test were recorded via infrared photobeams (Almatroudi et al., 2015). Illumination was 150 lux in the open arms and <1 lux in the closed arms. Mice were treated with saline, BU10119 (1 mg/kg), buprenorphine/naltrexone (1mg/kg) combination and diazepam (2mg /kg) 60 min prior to testing.

Light Dark Box (LDB)

The number of entries into and time spent in the lit compartment (400 lux), and the distance travelled during a 10 min LDB test were recorded via beam-breaks (Almatroudi et al., 2015). Mice were treated with saline, BU10119 (1 mg/kg), buprenorphine/naltrexone (1 mg/kg) combination and diazepam (2 mg /kg) 60 min prior to testing.

Conditioned place preference (CPP)

Place preference conditioning was conducted in a CPP chamber with an auto monitoring tracking system (Ethovision XT version 8.0, Tracksys, Nottingham, UK) as described previously (Almatroudi et al., 2015). Experiments were performed

between 09:00 hours and 16:00 hours under dim light (approximately 15 lux). During all test sessions, the time each mouse spent in each compartment was recorded. Mice were randomly assigned to treatment groups and the pairing was counterbalanced (i.e. within each treatment group equal numbers of mice were drug-paired to each compartment type). On days 1 and 2 mice were habituated to the entire chamber for 15 minutes (one session/day), during which baseline preference scores were taken. On days 3–8 mice were conditioned (40 minutes) to one of the two compartments, and daily sessions alternated between drug treatment and saline (In all treatment groups mice received both drug and saline). Mice were given buprenorphine (1 mg/kg), BU10119 (1mg/kg), morphine (10 mg/kg) or saline (0.9 % w/v). Where CCAM (3 mg/kg) was injected this was 24 hr before conditioning and mice were immediately returned to the home cage. After buprenorphine injection, the mice were transferred directly to the place preference box and at the end mice were returned to their home cage. Chamber floors and trays were removed and cleaned with ethanol 70% and left for 5 minutes for ethanol to evaporate before the next trial. On day 9, mice were not injected with saline or drugs. In a free-to-explore test, lasting 15 minutes, mice had free access to both compartments and their preference was determined by recording the time spent in the drug-paired chamber. Data are presented as preference for drug-paired side of CPP chamber, determined as the time spent in drug paired side minus time spent in drug paired side pre-conditioning (baseline).

Restraint stress

Mice in restraint-stressed groups were restrained in a well-ventilated modified 50 ml syringe for 2 h on 3 consecutive days from 09:00-11:00 hours (Sadler and Bailey,

2016). Stressed mice were weighed daily, monitored and scored for signs of stress. Non-stressed control mice were weighed daily and returned to their home cage.

Measurement of corticosterone level

All blood samples were collected from the lateral tail vein using the tail nick method and the minimal blood volume (~40 µl) collected in a heparinised capillary tube (Sadler and Bailey, 2013). Samples were taken at baseline, 24 h before the first restraint session, and immediately following the end of restraint stress (11:00 to 13:00 hours). Blood was collected in centrifuge tubes containing EDTA (final concentration in sample 3 µg/µl) and kept on ice until being centrifuged for 20 min at 4°C at 2000rcf. Plasma was taken and stored at -20°C until analysed using an ELISA kit (IBL International, Hamburg, Germany) to determine the level of corticosterone.

Statistical analysis

All data were analyzed using two-way repeated measures mixed model analysis or single measures one-way analysis of variance (ANOVA) followed by Unadjusted Least Significant Difference (ULSD) post hoc test (InVivoStat 2.3). Only planned pairwise tests were carried out and *p* values adjusted for multiple comparisons with Benjamin-Hochberg correction. In CCAM, buprenorphine and morphine CPP study unpaired Student's t-test was used. Values are reported as mean ± standard error of the mean (SEM) for each treatment group.

Results

Establishing the opioid receptor pharmacology of BU10119 *in vivo*

Pilot studies in mice revealed no overt signs of toxic effects of BU10119 at doses up to 10 mg/kg using a minimal numbers approach (n=3 per dose) (Hillhouse et al. 2016). The *in vivo* pharmacology of BU10119 was established using the warm water tail withdrawal test in adult male CD1 mice. BU10119 at 0.3, 1 and 3 mg/kg produced no significant antinociceptive action up to 4 h post-injection (Figure 2A; n=5 per group). Two-way repeated measures mixed model analysis revealed a significant interaction of Treatment*Time ($F_{(12,60)} = 19.35$, $p < 0.001$). Post-hoc testing showed that only buprenorphine (1mg/kg) produced a significant antinociceptive effect compared to saline treated controls, that peaked at 60 min post-administration ($p < 0.001$) and returned to baseline after 240 min.

To determine the κ -antagonist properties of BU10119 (0.3, 1 and 3mg/kg), its ability to block U50,488-induced antinociception was determined at 1, 8, 24 and 48 hours post-administration (Figure 2B; n=5 per group). Two-way repeated measures mixed model analysis revealed that there was a significant interaction of Treatment*Time ($F_{(28,140)} = 5.46$, $p < 0.001$). U50,488 produced a pronounced antinociceptive effect that was significantly reduced by BU10119 (1 and 3 mg/kg) at 1, 8 and 24 h post-administration ($p < 0.001$) but not at 48h. The high affinity, selective κ -antagonist norBNI (1 mg/kg) was able to block U50,488-induced antinociception at all-time intervals tested (all p 's < 0.001 , compared to U50,488).

In addition to κ -antagonist properties, BU10119 demonstrated μ -antagonist properties in the tail withdrawal assay (Figure 2C). Two-way repeated measures

mixed model analysis revealed that there was a significant interaction of Treatment*Time ($F_{(7,28)} = 18.68$, $p < 0.001$). Post-hoc testing showed that BU10119 (≥ 1 mg/kg) significantly blocked the antinociceptive effect of buprenorphine (1 mg/kg) ($p < 0.001$) and morphine (10 mg/kg) ($p < 0.001$) 60 min post-administration. The irreversible μ -antagonist CCAM (3 mg/kg), administered 24h before buprenorphine or morphine, was also able to block antinociception significantly (p 's < 0.001). Taken together these data show that, *in vivo*, BU10119 is a mixed κ/μ -antagonist with relatively short acting κ -antagonist activity (24-48 h).

Effects of BU10119 on locomotor activity

The open-field arena was used to assess the locomotor effects of BU10119 (0.3, 1 and 3 mg/kg) in CD-1 male mice. There were no significant effects of BU10119 on locomotion ($F_{(3,16)} = 1.65$, $p = 0.218$) (Figure 3). However, there was an apparent trend for increased locomotion at 3 mg/kg. For this reason, in subsequent behavioural tasks, BU10119 was investigated at 1 mg/kg alone.

Effects of BU10119 in the conditioned place preference (CPP) task

One-way ANOVA revealed a significant effect of Treatment on the preference for the drug paired compartment of the CPP chamber ($F_{(5,48)} = 6.78$, $p < 0.0001$). Mice receiving 1 mg/kg of buprenorphine exhibited significant conditioned place preference compared to saline treated mice ($p < 0.01$, $n = 9$ per treatment group, Figure 4A).

BU10119 at the same dose appeared to increase preference for the drug-paired side although this effect was not significant ($p = 0.08$). The irreversible μ -antagonist CCAM (3 mg/kg), was administered 24 h before saline, buprenorphine or BU10119.

Interestingly, CCAM appeared to reduce the effects of BU10119 in the CPP chamber

($p=0.058$). However, CCAM failed to block or to reduce the effects of buprenorphine in the CPP chamber ($p=0.914$). CCAM alone was neither rewarding nor aversive. There were no significant differences in baseline times, determined pre-conditioning, between any of the treatment groups.

In a subsequent experiment (Figure 4B), CCAM (3mg/kg) was able to significantly block the rewarding effects of morphine in the CPP task. One way ANOVA revealed that there was a significant effect of treatment on the preference for the drug paired side ($p<0.05$). However, CCAM (3mg/kg) was again not able significantly to reduce the time spent in the drug paired side for buprenorphine (1mg/kg) ($p = 0.3838$, $n = 8$ per treatment group, unpaired t-test). Taken together these data show that in contrast to the tail withdrawal assay, BU10119 may have some weak μ -agonist activity in the CPP task. The apparent increase in preference for the drug paired side shown by BU10119, although not significant, may suggest weak μ -agonist properties that are blocked by the irreversible μ -antagonist CCAM. Interestingly, buprenorphine's rewarding effects in the CPP task are not apparently mediated via its partial μ -agonist activity.

Effects of BU10119 on depression- and anxiety related behaviours

The antidepressant-like effects of BU10119 were assessed using the forced swim test (FST) and the novelty-induced hypophagia task. One-way ANOVA revealed a significant effect of Treatment on the time spent swimming ($F_{(3, 36)}=6.58$, $p<0.001$) and immobile ($F_{(3, 36)}=7.02$, $p<0.001$) during the last 4 minutes of a forced swim test session (Figure 5A). Post hoc analysis revealed that BU10119 (1mg/kg),

combination buprenorphine/naltrexone (1 mg/kg) and fluoxetine (20 mg/kg) increased the time spent swimming and decreased the time spent immobile compared to saline treated controls (n=10 per treatment group, all p's <0.001).

In the novelty-induced hypophagia task (Figure 5B), two-way repeated measures mixed model analysis of the latency to drink times revealed significant main effects of Treatment ($F_{(4,75)}=6.13$, $p<0.001$) and a significant Treatment*Environment interaction ($F_{(4,75)}=5.92$, $p<0.001$). The novel cage was shown to be aversive as saline control treated mice showed a significant increased latency to drink milk in the novel cage (latency= 7.32 ± 0.94 min) versus the home cage (latency= 0.48 ± 0.10 min, $p<0.001$). Within treatment, post-hoc comparisons to saline treated controls revealed that all drug treated groups decreased the latency to drink milk in the novel cage (n=10 per treatment group, all p's <0.001).

In the EPM, one-way ANOVA, showed significant effects of Treatment on the time spent in ($F_{(3, 36)}=3.29$, $p< 0.05$) and number of entries into ($F_{(3, 36)}=3.89$, $p<0.01$) the open arms (Figure 6 (A–C), n=10 per group). Post hoc comparisons to saline treated controls revealed that only the benzodiazepine diazepam (2 mg/kg) significantly increased these parameters ($p<0.01$). Interestingly, both buprenorphine/naltrexone (1 mg/kg) combination and BU10119 (1 mg/kg) did not show any significant changes in behaviours in the EPM. Total ambulation in the EPM was not affected by drug treatment ($F_{(3,36)}=1.15$ $p=0.342$), showing an absence of any sedative effects. Furthermore, in the LDB there were no significant effects of either BU10119 or combination buprenorphine/naltrexone (Figure 6(D–F), n=18 per group). One-way

ANOVA revealed a significant main effect of Treatment on the time spent in the light ($F_{(3, 60)}=3.59$, $p < 0.01$) and dark ($F_{(3, 60)}=3.59$, $p < 0.01$) compartment of the LDB. However, within-treatment analysis to saline controls showed that only diazepam (2 mg/kg) significantly increased the total time spent in the lit compartment ($p < 0.01$). As with the EPM, total ambulation in the LDB was not significantly affected by drug treatment ($F_{(3,60)}= 1.26$, $p = 0.29$).

Stress induced analgesia and elevation in corticosterone level

To assess whether BU10119 or combination buprenorphine/naltrexone could block the effects of stress in adult male mice, stress-induced analgesia was assessed in the warm water tail-withdrawal assay (Figure 7A). Two-way repeated measures mixed model analysis revealed a significant interaction of Treatment*Time ($F_{(12,75)} = 23.3$, $p < 0.001$). Exposure to acute restraint (1 x 2h restraint) or 3 days repeated restraint stress (3 x 2h restraint) produced a significant stress-induced analgesia evident as an antinociceptive effect in restraint stressed mice compared to non-stressed controls ($p < 0.001$, $n = 6$ per treatment group). Pre-treatment with BU10119 (1mg/kg), combination buprenorphine/naltrexone (1mg/kg) or the selective κ -antagonist norBNI (10mg/kg) blocked stress-induced analgesia ($p < 0.001$), compared to respective acute or repeated stressed saline control groups. Furthermore, there was no significant difference between the baseline tail withdrawal latency in non-stressed control groups in the first and third day of experiment ($p = 0.3344$).

Blood samples were collected at baseline (24 h pre-stress) and after 3 days repeated restraint stress, from the same animals, for analysis of corticosterone levels (Figure 7B). Two-way repeated measures mixed model analysis revealed that there was a significant effect of stress ($F_{(1,21)}=62.62$, $p<0.001$), no significant effects of treatment ($F_{(4,25)}=1.2$, $p<0.3299$) but a significant interaction Stress * Treatment ($F_{(4,21)}=13.14$, $p<0.001$) on plasma corticosterone levels. Post-hoc comparisons revealed no significant difference in baseline plasma corticosterone levels in all mice prior to treatment. However, following 3 days of repeated restraint stress, there was a significant effect of stress in all drug-treated groups compared to non-stressed saline-injected controls (all $p\leq 0.01$) but none of the drug treatments were able to block stress-induced increases in plasma corticosterone (all $ps > 0.05$).

Discussion

We have previously shown that the combined administration of buprenorphine and naltrexone produces antidepressant-like effects in mice (Almatroudi et al., 2015). BU10119 is a recently reported novel compound with an *in vitro* pharmacology that resembles the combination of buprenorphine/naltrexone: high affinity/ zero efficacy at κ -opioid receptors, high affinity/ little efficacy at μ -opioid receptors and a weak partial agonist profile at NOP receptors (Cueva et al., 2015). Here we report for the first time the *in vivo* pharmacology of BU10119 in adult male CD1 mice. The data shows BU10119 has a mixed κ/μ -antagonist profile and the κ -antagonist effects are relatively short-acting with no activity evident at 24-48 h post-administration. We also show for the first time the antidepressant-like properties of BU10119 in the forced swim test and the novelty-induced hypophagia task. Importantly, BU10119 is without significant locomotor effects at the doses used and has no significant rewarding effects in the conditioned place preference task. Finally, we have shown that BU10119 is able to block stress-induced analgesia, although it did not reduce stress-induced increases in plasma corticosterone.

In the tail withdrawal assay, BU10119 (0.3 to 3 mg/kg) was without significant analgesic effects suggesting it has no agonist efficacy at any of the opioid receptors at the doses studied. Furthermore, BU10119 (1mg/kg and 3 mg/kg) was able to significantly reduce the analgesic effects of the κ -agonist U50,488 and of the μ -agonists buprenorphine and morphine. These data clearly indicate that BU10119 has a mixed κ/μ -antagonist profile *in vivo*. These results are consistent with *in vitro* studies using the rodent vas deferens which found that BU10119 acts as a κ -opioid

receptor antagonist, with an average pA₂ value of 9.831 (9.084-10.58), and a competitive reversible antagonist at μ -opioid receptors with a pA₂ value of 10.08 (9.847-10.310) (Ridzwan, 2012). We have previously shown that naltrindole-derived compounds with a similar mixed κ/μ -antagonist profile may have antidepressant and anxiolytic potential (Casal-Dominguez et al., 2013). However, those compounds had long lasting κ -antagonist effects; 21-35 days following a single injection (Casal-Dominguez et al., 2013). Experiments in the tail-withdrawal assay show the time course of BU10119's κ -antagonist effects. Interestingly, BU10119's effects in the tail withdrawal assay are not evident 24-48h post-administration making it a relatively short-acting κ -antagonist. This is slightly longer than the duration of action of combination buprenorphine/naltrexone or naltrexone alone which have a duration of effect <24h (Almatroudi et al. 2015) but shorter than the prototypical κ -antagonist norBNI (Casal-Dominguez et al., 2013).

While BU10119 (1mg/kg) was without any agonist efficacy in the tail-withdrawal assay, or in *in vitro* GTP γ S assays (Table 1), the conditioned place preference task revealed that BU10119 may be a weak partial μ -agonist. Measures of partial agonist activity in GTP γ S assays are dependent on specific experimental design such as expression levels. Indeed, naltrexone has been reported to have partial μ -agonist activity under conditions of high receptor expression (Kelly et al., 2015). BU10119 appeared to increase the time spent in the drug-paired compartment of the CPP apparatus, although not to the same extent as buprenorphine, and this effect was not statistically significant. Interestingly, the irreversible μ -antagonist CCAM (3mg/kg) was able to reduce the apparent rewarding effects of BU10119 suggesting that these effects are mediated by BU10119 activating μ -opioid receptors. We confirmed that

CCAM was acting to block μ -opioid receptors as it reduced the ability of morphine to produce conditioned place preference in a separate experiment. Interestingly, in both CPP experiments performed here the rewarding properties of buprenorphine were not reversed by CCAM. This is consistent with findings in μ -receptor knockout mice that buprenorphine maintains its rewarding properties (Ide et al., 2004). However, in another study using different CPP protocols and different μ -opioid receptor knockout mice, conditioned place preference to buprenorphine was not demonstrated (Marquez et al., 2007). Ide et al. (2004) also showed that the nonselective opioid antagonist naloxone, and to a lesser extent the δ -antagonist naltrindole, and the κ -antagonist norBNI were able to reduce buprenorphine conditioning suggesting that multiple opioid receptors are involved in mediating the rewarding effects of buprenorphine. It is interesting that the antidepressant drug fluoxetine has also been shown to produce conditioned place preference (Collu et al., 1997), suggesting that perhaps any drug with a positive, stress-relieving action may somewhat increase preference for the drug paired compartment.

BU10119 shows antidepressant-like activity in both the forced swim test and the novelty-induced hypophagia test. The acute administration of the SSRI fluoxetine has been shown previously to reduce the time spent immobile in the mouse forced swim test; a widely used behavioural screen for antidepressant efficacy (Lucki, 1997, Cryan et al., 2002). Our data show that BU10119 produces effects equivalent to fluoxetine in the forced swim test. We have previously reported the effects of norBNI (10 mg/kg) in adult male CD-1 mice in the forced swim test 6, 13 and 20 days post-administration (Casal Dominguez et al., 2013) and 24-48 h post administration (Almatroudi et al., 2015). In both these experiments, norBNI produced responses equivalent to

fluoxetine. The novelty-induced hypophagia task is a procedure developed to assess anxiety-related behaviours using a conflict based approach-avoidance task that is sensitive to a range of anxiety-relieving drugs including chronic, but not subchronic, administration of fluoxetine (Dulawa and Hen, 2005). Here we have shown that acute fluoxetine (20 mg/kg) delivered by intraperitoneal injection, 1 h prior to testing in both the home and novel cage, did reduce the latency to feed in the novel cage in CD-1 mice, as we have previously reported (Almatroudi et al. 2015). Several procedural variations could account for these observations including mouse strain and route of administration: Dulawa and Hen (2005) examined a range of doses of fluoxetine (0-25 mg/kg) delivered via drinking water to BALB/cJ mice for 4-5 days (subchronic) or for 28/29 days (chronic). Furthermore, Dulawa and Hen administered fluoxetine throughout the training period whereas fluoxetine was only administered here during the testing period. The behavioural effects of BU10119 in both the forced swim test and novelty-induced hypophagia tasks resembled that of the SSRI fluoxetine and the combination of buprenorphine/naltrexone suggesting that it has antidepressant-like activity.

Interestingly, BU10119 was without any appreciable effects in the elevated plus maze and light dark box tasks. This is similar to our previous studies with combination buprenorphine/naltrexone and naltrexone alone (Almatroudi et al., 2015). This is perhaps surprising given that dynorphin has been shown to induce significant anxiogenic like effects in mice in the elevated plus maze (Narita et al., 2005) while high affinity selective κ -antagonists, norBNI and JDTic, have been shown to produce anxiolytic like effects (Knoll et al., 2007). A lack of anxiolytic-like effect in the elevated plus maze may be related to the duration of κ -antagonist

effects. We have previously shown that long-acting mixed μ/κ -antagonists, and the selective κ -antagonist nor-BNI, have anxiolytic-like activity in these tasks when tested 7 and 14 days post-administration (Casal-Dominguez et al. 2013). BU10119, naltrexone and combination buprenorphine/naltrexone are relatively short-acting κ -antagonists and don't display this anxiolytic-like effect in the elevated plus maze and light dark box (Almatroudi et al., 2015). Similar findings have been reported with the short-acting κ -antagonists zyklophin and LY2444296 which have effects in a novelty-induced hypophagia paradigm but no anxiolytic-like activity in the elevated plus maze (Huang et al., 2016). This was in contrast to norBNI which they showed to be effective in both behavioural paradigms, as we have shown previously, also in CD1 mice (Casal Dominguez et al., 2013; Almatroudi et al. 2015). The duration of activity of κ -receptor antagonists has been demonstrated to correlate with c-Jun N-terminal kinase-1 activation (Melief et al. 2011). It is not clear whether long duration of κ -antagonist action is required to produce behavioural effects in the elevated plus maze as other short-acting κ -antagonists, AZ-MTAB, and LY-DMPF, have been shown in prenatally stressed rats to exhibit anxiolytic type responses (Peters et al., 2011). The absence of a robust anxiolytic like effect of BU10119 in naïve mice may be due to the fact that the elevated plus maze and light dark box are not sufficiently stressful paradigms to activate dynorphin release and alter anxiety behaviours (McLaughlin et al., 2006a, Shirayama et al., 2004, Wittmann et al., 2009).

The phenomenon of stress-induced analgesia is an endogenous protective mechanism that occurs in response to stressful stimuli and involves activation of the descending inhibitory pain pathways (Butler and Finn, 2009). In this study we have used the warm-water tail withdrawal assay to assess stress-induced analgesia. This

model may be complicated by stress-induced changes in skin temperature arising from vasoconstriction, but this does not preclude an interpretation of stress-induced analgesia (Butler and Finn, 2009). Acute stress has been shown to produce antinociception whereas prolonged or repeated exposure to the stress results in tolerance or even hyperalgesic responses (Seo et al., 2011, Seo et al., 2006, Gamaro et al., 1998). Different types of stressors including restraint, cold-swim or presence of a predator can elicit opioid mediated stress-induced analgesia, which activates distinct neuroanatomical structures (Keay and Bandler, 2001). In this study, we show that both acute and repeated restraint stress resulted in stress-induced analgesia that was blocked by pre-treatment with BU10119 and the combination buprenorphine/naltrexone. Restraint stress-induced analgesia is blocked by pre-treatment with the κ -antagonist norBNI in female rats (Botelho et al., 2010) and in mice exposed to forced swim stress (McLaughlin et al., 2003). Interestingly, forced swim stress-induced analgesia has been shown to be absent in prodynorphin $-/-$ mice (McLaughlin et al., 2003) but is not altered in κ -receptor knockout mice (Contet et al., 2005). As these authors discuss, κ -receptor and prodynorphin knockouts are not equivalent animal models since the prodynorphin gene encodes a number of opioid peptides that activate opioid receptors (Contet et al., 2005). There are also differences in these studies in the application of the forced swim stress and in the methodology to assess stress-induced analgesia. Here, we have demonstrated that restraint stress-induced analgesia is blocked by κ -antagonists suggesting the possibility that these compounds may have a role as prophylactic stress treatments (Van't Veer and Carlezon, 2013).

Interestingly, BU10119 was not able to block stress-induced increases in plasma corticosterone. There are contradictory reports in the literature about the impact of κ -receptors on plasma corticosterone levels. McLaughlin et al. (2006) used repeated forced swim stress in C57BL/6 mice which produced a 3-fold increase in plasma corticosterone. They went on to show that following pretreatment with the κ -antagonist norBNI, and in prodynorphin knockout mice, swim stress still produced an increase in plasma corticosterone. However, Wittmann et al. (2009) reported that basal corticosterone serum levels were reduced in prodynorphin knockout animals and in wildtype mice pretreated with 10 mg/kg norBNI. In food restricted rats elevated plasma corticosterone levels were reduced by treatment with norBNI (Allen et al., 2013). However, swim stress in rats induced elevated plasma corticosterone that was not blocked by norBNI treatment (Polter et al., 2014). Contet et al. (2005) used a swim stress in warm water in κ -receptor knockout mice and suggested the dissociation of stress-induced analgesia and stress-induced increases in plasma corticosterone levels. These apparently contradictory findings could be explained in part by the different stressors used which would likely implicate different circuitry in the stress coping behaviours (Pacák and Palkovits, 2001). It is noteworthy that in all of these studies, stress-induced behaviours were blocked by treatment with norBNI, or in dynorphin $-/-$ mice, even though stress-induced corticosterone levels may or may not have been affected. Thus κ -antagonists may have therapeutic potential in treating stress-induced behaviours independent of effects on corticosterone levels (Carroll and Carlezon, 2013, Van't Veer and Carlezon, 2013).

Naltrexone is a relatively non-selective opioid receptor antagonist with higher affinity for μ than κ -opioid receptors. In the UK it is licensed as an abstinence promoter

(Rosner et al., 2010). However, naltrexone is associated with aversive side effects. Combining naltrexone (50 mg daily) with buprenorphine (4 mg daily) has been shown to improve patient compliance in the treatment of opioid dependence, as this improves the dysphoria associated with drug withdrawal (Gerra et al. 2006). However, the tolerability of naltrexone has been shown to be significantly improved when given at lower doses and low dose naltrexone (< 4mg daily) is increasingly being used for the treatment of a number of chronic conditions (Segal et al., 2014, Younger et al., 2014). Low dose naltrexone has recently been trialled in a small cohort of major depressive disorder patients, maintained on antidepressant therapy but experiencing a relapse (Mischoulon et al., 2017). This study investigated whether augmentation of dopaminergic antidepressant regimens (mostly bupropion) with 1mg naltrexone for 3 weeks would reduce Hamilton Depression Scale (HAM-D17) scores by 50% compared to baseline. There was an apparent effect of low dose naltrexone although it was not significantly different from placebo in this small cohort study (Mischoulon et al., 2017). The potential of opioid antagonist mechanisms for the treatment of depression is also evident in the combination of buprenorphine with the μ -antagonist samidorphan producing a functional mixed μ/κ -antagonist (Ehrich et al., 2015). Following 7 days of once daily buprenorphine/samidorphan at a 1:1 ratio, patients with treatment resistant major depressive disorder showed a significant improvement in HAM-D17 total score (Ehrich et al., 2015). These clinical findings, coupled with our preclinical data, suggest that molecules like BU10119 with a pharmacology resembling combination buprenorphine/naltrexone could have significant potential in the treatment of depression.

Figures

Figure 1 Chemical structures of buprenorphine and BU10119.

Figure 2 Effects of BU10119 in adult male CD1 mice in the warm water tail withdrawal assay. The time course of the experiments is indicated and tail withdrawal latencies are expressed as % maximum possible effect (%MPE). (A) Antinociceptive effects of buprenorphine (1 mg/kg) and BU10119 (0.3, 1 and 3 mg/kg). *** $p < 0.001$ as compared between buprenorphine and all other groups. (B) Antinociceptive effects of the κ -agonist U50,488 (10 mg/kg) were blocked by BU10119 (1 and 3 mg/kg) and by norBNI (1 mg/kg). *** $p < 0.001$ as compared to BU10119 (1 and 3 mg/kg) and norBNI (1mg/kg), ### $p < 0.001$ as compared between all groups and norBNI (1mg/kg). (C) Antinociceptive effects of buprenorphine and morphine at 60 min post-administration were blocked by BU10119 (1 mg/kg) and by the irreversible μ -antagonist CCAM (3 mg/kg). *** $p < 0.001$ compared to buprenorphine, ### $p < 0.001$ compared to morphine. All values are mean \pm SEM, $n = 5$ per group, separate experimental groups in each figure.

Figure 3 Locomotor activity in the open field in adult male CD1 mice treated with BU10119 (0.3, 1 and 3mg/kg). All values are mean \pm SEM, $n = 5$ per group. No statistically significant effects on locomotion were observed.

Figure 4 Conditioned place preference to buprenorphine (BUP, 1mg/kg), BU10119 (1mg/kg) and morphine (10 mg/kg). Two separate experiments were conducted. (A) BU10119 did not produce significant conditioned place preference. Conditioned place preference to buprenorphine was not blocked in the presence of the

irreversible μ -antagonist CCAM (3mg/kg). (B) The activity of CCAM as a μ -antagonist was confirmed by its ability to block morphine-induced conditioned place preference. Data are presented as preference for drug-paired side of CPP chamber, determined as the time spent in drug paired side (post-conditioning) minus time spent in drug paired side pre-conditioning (baseline). All data points are mean \pm SEM. (A) ** $p < 0.01$ compared to saline, $n=9$ per treatment group. (B) * $p < 0.05$ compared to morphine, $n=8$ per treatment group.

Figure 5 Effects of BU10119 (1mg/kg) in adult male CD1 mice in the forced swim test (A) and in the novelty-induced hypophagia task (B). The SSRI fluoxetine (20mg/kg) was administered as a positive control. The effects of combination of buprenorphine with naltrexone (both at 1mg/kg, BUP/NTX) and the κ -antagonist norBNI are also shown. (A) The total time spent swimming, climbing or immobile during the last 4 min of the forced swim session are shown. (B) The latency to drink milk in both the home and novel cage environments is shown. All values are the mean \pm SEM ($n=10$ per group, separate experimental groups in each figure). *** $p < 0.001$ as compared to saline, ### $p < 0.001$ for comparison between groups.

Figure 6 Effects of BU10119 (1mg/kg) in adult CD1 male mice in the elevated plus maze (A–C) and in the light-dark box (D–F). The effects of combination of buprenorphine with naltrexone (both at 1mg/kg, BUP/NTX) are also shown. The benzodiazepine diazepam (2 mg/kg) was used as a positive control. The time spent in the open arms (A), number of entries into the open arms (as a percentage of the total entries into open and closed arms) (B) and total ambulation (C) in the elevated plus maze are shown ($n=10$ per group). The time spent in the light box (D), in the

dark box (E) and total ambulation (F) in the light-dark box are shown (n=18 per treatment group, n=10 for BU10119). All values are the mean \pm SEM. **p< 0.01 compared to saline.

Figure 7 Ability of BU10119 (1mg/kg), combination buprenorphine/naltrexone (both at 1mg/kg, BUP/NTX) and the κ -antagonist norBNI to block stress-induced effects. Drug treatments were administered 1h prior to acute restraint stress (1 x 2h, Day 1) and daily repeated restraint stress (3 x 2h, Day 3) in adult male CD1 mice. (A) Stress-induced analgesia was evident, in the warm-water tail withdrawal assay, as increased % maximum possible effect (%MPE). Test latencies were assessed immediately following the restraint session. ***p< 0.001 compared to all groups within the same day. (B) Blood samples were taken at baseline and immediately following the last session of restraint to assess plasma corticosterone. All blood samples were taken during the light phase 11:00-13:00 h. ***p<0.001, compared to non-stress saline post day 3.## p<0.01,### p<0.001, compared to baseline for the same treated group. All values are the mean \pm SEM (n=6 per group, separate experimental groups in A and B).

Figure 1

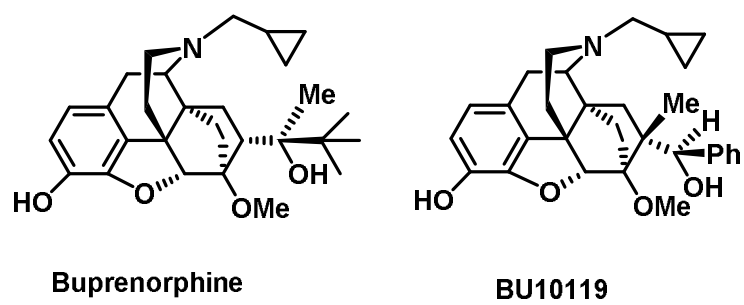


Figure 2

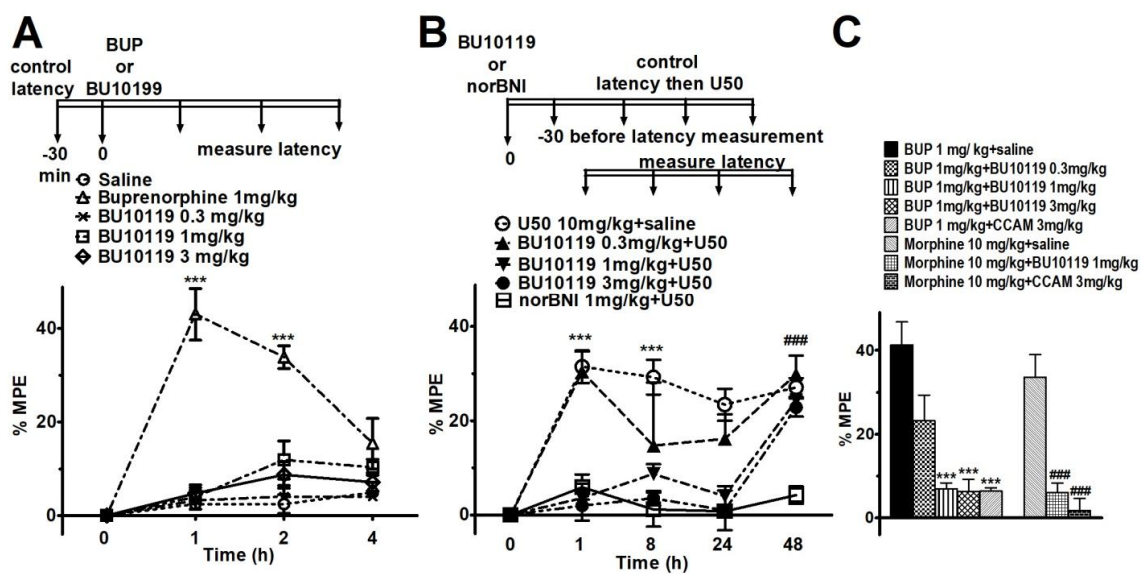


Figure 3

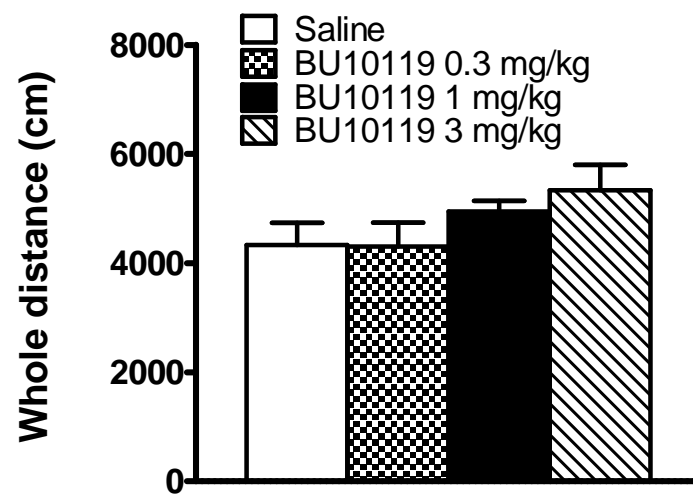


Figure 4

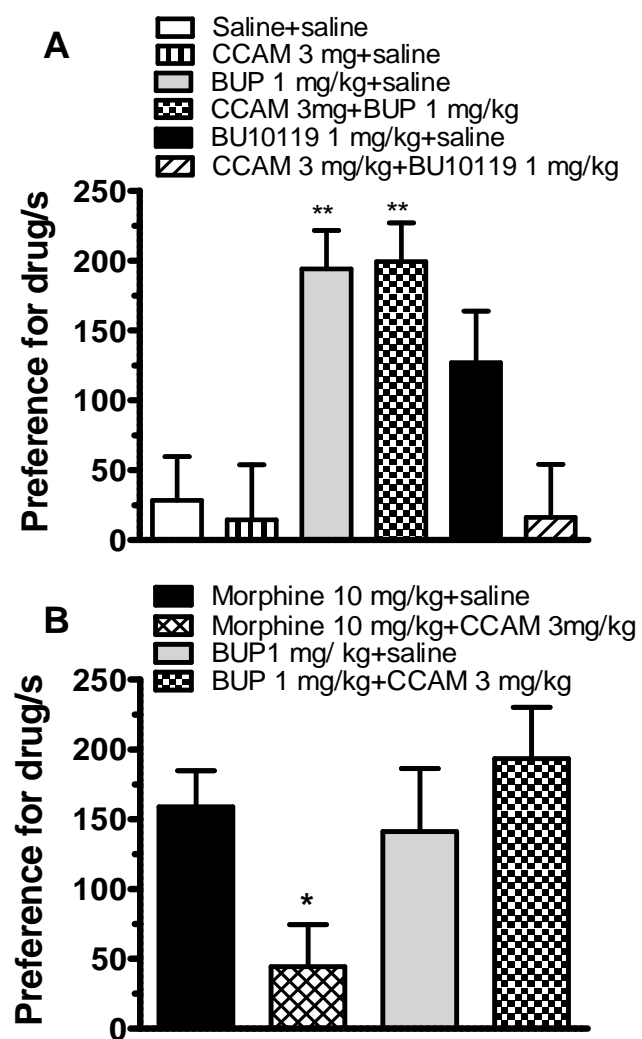


Figure 5

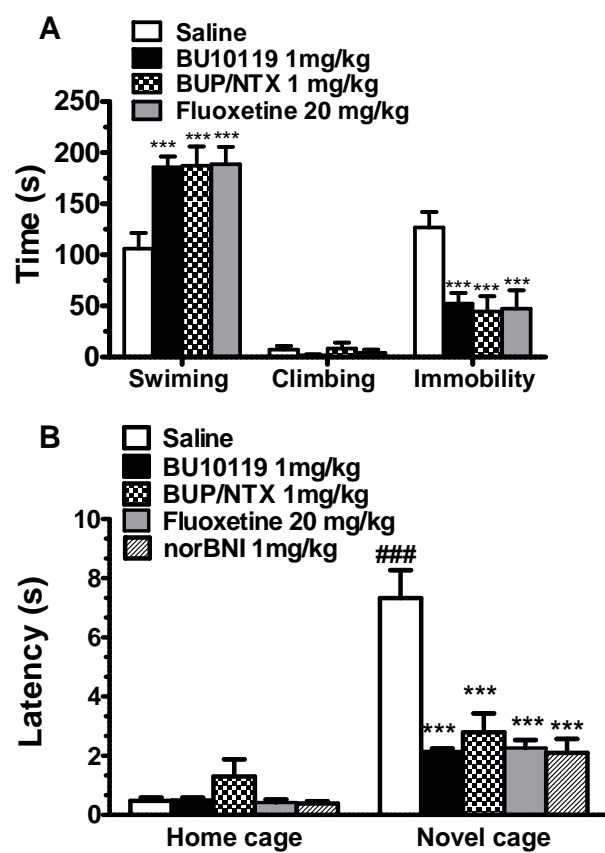


Figure 6

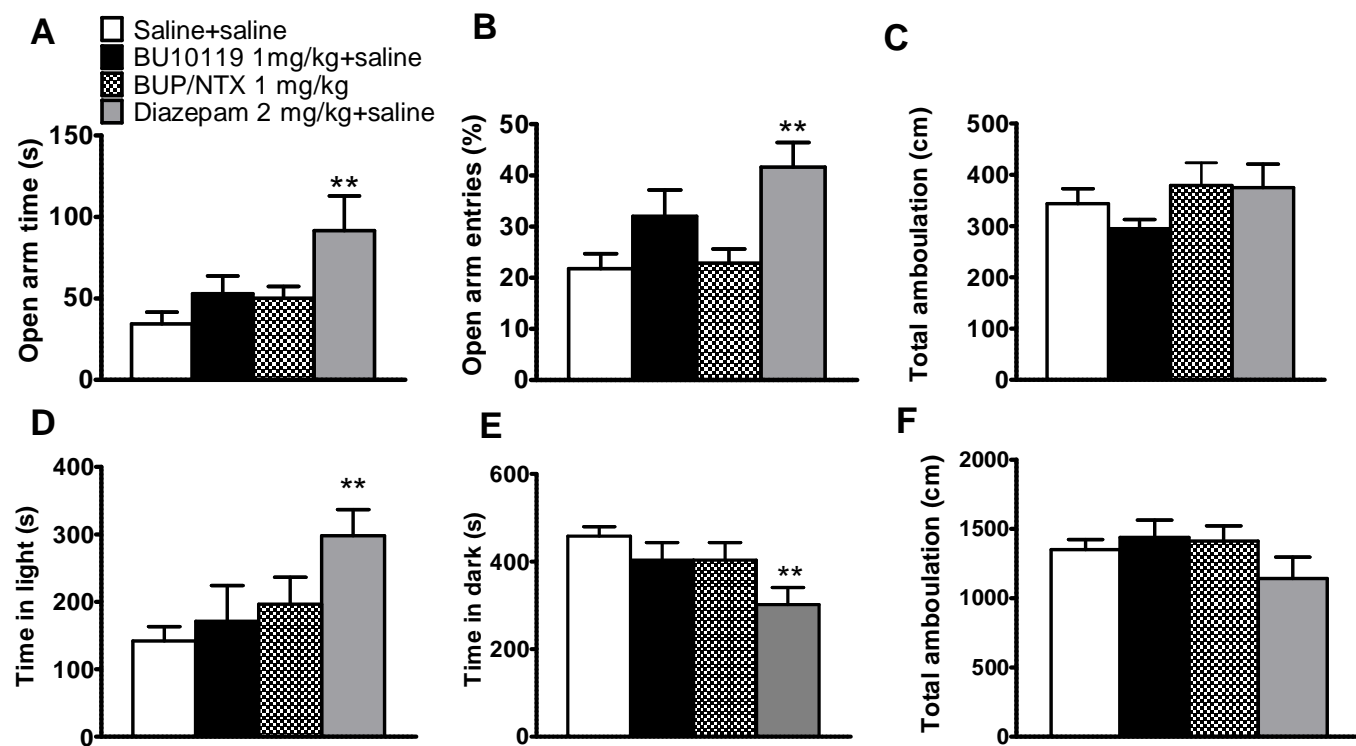
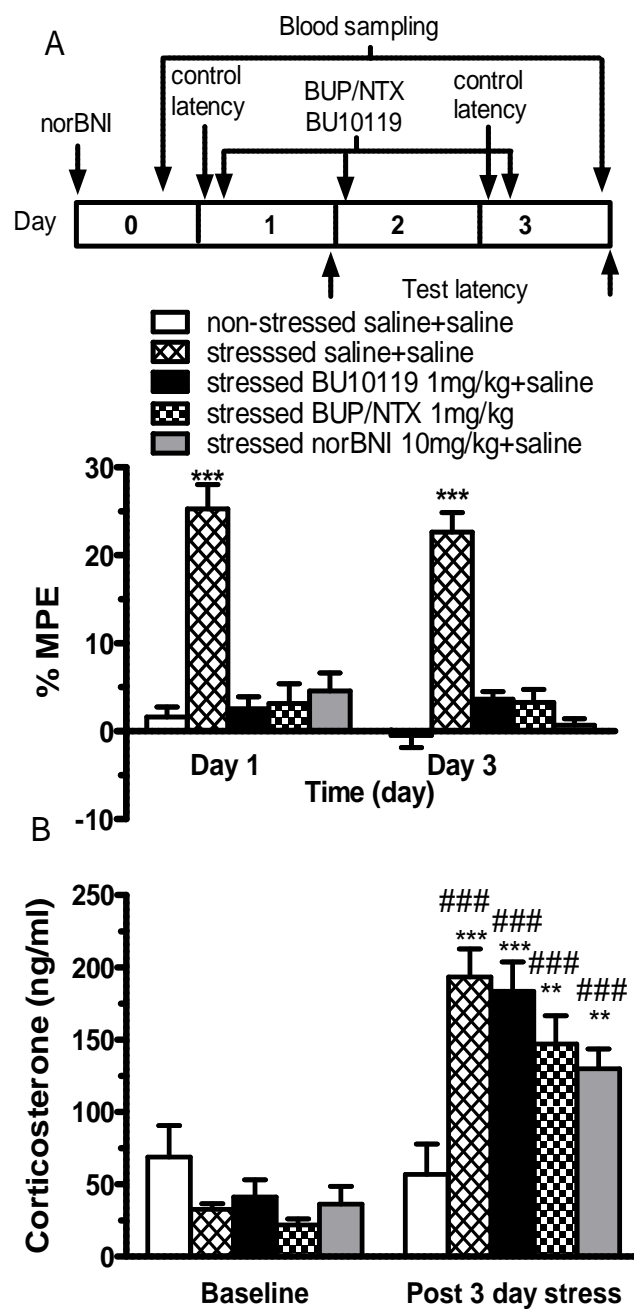
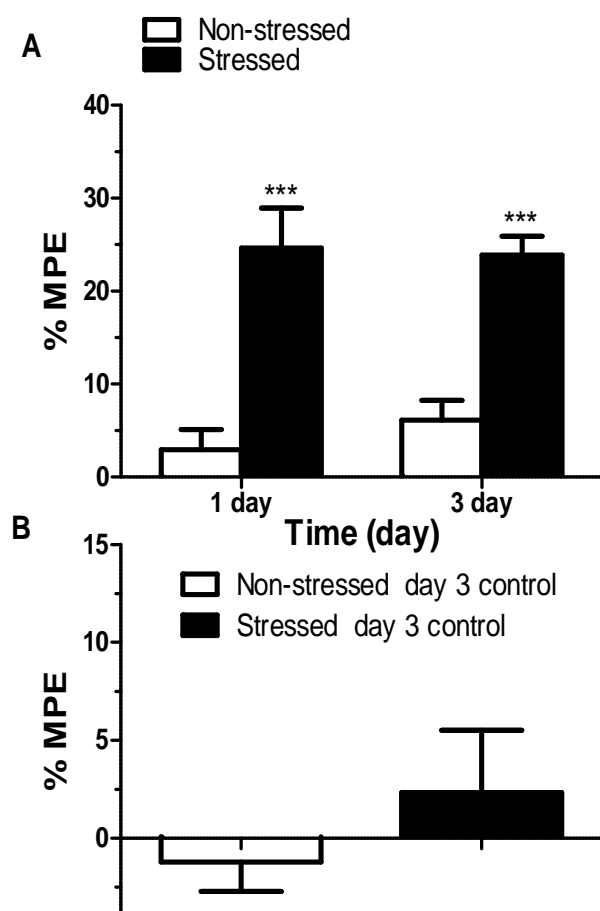


Figure 7



Supplementary figure 1



Supplementary figure 1 (A) Effect of 1 day and 3 day restraint stress (09:00-11:00) on stress-induced analgesia in CD1 male mice. (B) Baseline latency on day 3. Results are expressed as mean \pm SEM, n=4. ***p<0.001 as compared to non-stressed controls. Analysis done was repeated measures mixed model analysis using InVivo Stat software.

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